

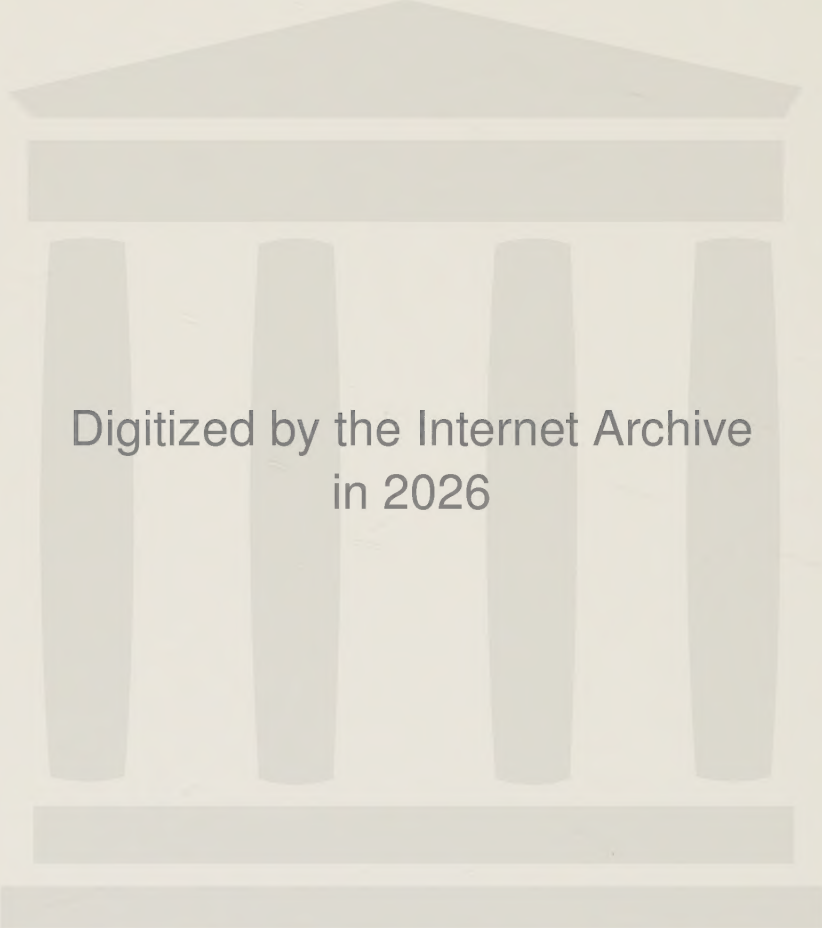


Wound healing and granulation tissue formation after defibrinogenation

An experimental study in the rabbit

By Stephan Brändstedt

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WOUND HEALING AND GRANULATION TISSUE FORMATION

AFTER DEFIBRINOGENATION

An experimental study in the rabbit

by

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Articles I-V	37-91

The present dissertation is based on the following publications,
which will be referred to in the text by their Roman numerals:

- I WOUND HEALING AND FORMATION OF GRANULATION TISSUE IN NORMAL
AND DEFIBRINOGENATED RABBITS. AN EXPERIMENTAL MODEL AND
HISTOLOGICAL STUDY.

In collaboration with F Rank and P S Olson

Eur Surg Res 12: 12-21, 1980

- II EFFECT OF DEFIBRINOGENATION ON WOUND STRENGTH AND COLLAGEN
FORMATION. A STUDY IN THE RABBIT.

In collaboration with P S Olson

In press. Acta Chir Scand, 1980

- III CELL COMPOSITION OF GRANULATION TISSUE IN DEFIBRINOGENATED
RABBITS.

In collaboration with F Rank, P S Olson and J Ahonen

In press. Acta Chir Scand, 1980

- IV EFFECT OF DEFIBRINOGENATION ON COLLAGEN SYNTHESIS IN
GRANULATION TISSUE.

In collaboration with P S Olson and J Ahonen

In press. Acta Chir Scand, 1980

- V LACK OF INFLUENCE ON COLLAGEN ACCUMULATION IN GRANULATION
TISSUE WITH "DELAYED" DEFIBRINOGENATION. A STUDY IN THE
RABBIT.

In collaboration with P S Olson

In press. Acta Chir Scand, 1981

INTRODUCTION

The first events to follow physical injury are haemorrhage and exudation. Subsequent coagulation produces a fibrinous framework that attaches to the wound surfaces and fills the wound space. Later, cells invade the wound area, some of them to remove debris, others to synthesize the various connective tissue elements and thereby to restore continuity and strength.

The healing of a wound is a continuous process in which, however, different phases can be recognized. First comes an inflammatory reaction, elicited by the injury, followed by proliferation of fibroblasts and vessels and finally maturation of the connective tissue with structural alteration of the collagen deposit.

The initial inflammatory reaction is of importance for the subsequent repair process, something that was first recognized by Carrell in 1921. There seems to be an optimal degree of inflammation (Ross, 1972). If no inflammation occurs, there is no reparative process and if the inflammatory reaction is depressed, repair is slow. On the other hand, if inflammation is marked, repair may also be delayed. Excessive inflammation increases tissue damage and causes breakdown of old and newly formed tissue. Continuing inflammation usually leads to excessive scar formation.

The factors by which inflammation mediates its effect on the subsequent repair process are largely unknown. The inflammatory cellular response seems to be of importance for fibroblast activation. Studies by Leibovich & Ross (1975) demonstrated that monocytes / macrophages are necessary both for wound debridement and for fibroblast proliferation. Heppleston & Styles (1967), Aalto et al. (1976) and Aho & Kulonen (1977) have demonstrated a factor released from macrophages activated by silica, which stimulates

collagen synthesis *in vitro*. This factor has been ascribed a role in chronic fibrotic diseases such as silicosis. Its role in granulation tissue formation is so far speculative. A platelet derived factor released during the clotting process that initiates fibroblast cell replication *in vitro* has been demonstrated by Kaplan et al. (1979). Platelet factors have also been attributed a potential role in angiogenesis since a stimulatory effect on the proliferation of smooth muscle cells could be demonstrated *in vitro* (Ross et al., 1974; Wall et al., 1978a). Not only cell derived factors but also fibrin and thrombin have growth promoting properties *in vitro* (Chen & Buchanan, 1975; Teng & Chen, 1975; Pohjanpelto, 1977; Bruhn et al., 1978; Pohl et al., 1979).

Fibrin is a major component of the early inflammatory exudate, constituting about 20% of the formed elements. The presence of fibrin in a wound has long been known. Marchand (1901) established a connection between the fibrin deposited in a wound and the coagulating protein of the blood.

Most of the fibrin deposition takes place early after injury. There is, however, some evidence that fibrin in the wound is remodelled later in the process. Stearns (1940) following the development of connective tissue *in vivo* in rabbits through transparent ear chambers observed that the fibrin strands during the first days after injury gradually changed appearance and became more distinct and solid. ¹²⁵I labelled fibrinogen administered intravenously at different intervals after wounding has been found to accumulate in developing granulation tissue in significant amounts up to two weeks postoperatively (Brändstedt, to be published).

Co-incident with collagen deposition fibrin is removed. Leucocytes and macrophages are known to ingest fibrin (Barnhart, 1965; Jørgensen et al., 1967). In experimental wounds deprived of macro-

phages there is a significant increase in fibrin content (Leibovich & Ross, 1975). The fibrinolytic activity of these cells, however, is weak compared with the fibrinolytic activity elicited by the plasminogen activators of the advancing capillaries (Kwaan & Astrup, 1964). Todd (1959) could identify the vascular endothelial cells as the source of the plasminogen activator, that regulates the fibrinolysis in tissues (Sherry, 1966). The ultimate process of fibrin removal in connective tissue repair seems to be executed mainly by the advancing capillaries (Kwaan, 1965).

Fleisher & Loeb suggested as early as 1915 that fibrin formation and resolution were important factors in the regulation of tissue repair. This aspect, however, remained unnoticed until Astrup (1956) adopted the concept which has since found support in some experimental studies. Benzer et al. (1963) showed that increased fibrinolytic activity in the blood, induced by streptokinase given during the two first postoperative days, impaired wound strength development. Correspondingly improved wound healing was observed when the fibrinolytic activity in the blood was decreased by protease inhibitors (Benzer et al., 1964; Flandre et al., 1966). Kwaan & Astrup (1969) showed that granulation tissue formation was enhanced by local application of protease inhibitors. Heparin administration has been demonstrated in experimental studies to decrease wound strength development (Ludewig et al., 1970; Thompson, 1972) and collagen synthesis (Ludewig et al., 1970). Heparin has also experimentally been used to prevent the formation of peritoneal adhesions (Lehman & Boys, 1940; Knightly et al., 1962; James et al., 1965). In recent years new interest has been brought into this field by the introduction of defibrinogenating agents. Holt et al. (1970), Silbermann & Kwaan (1971) and Olsson et al. (1973) found delayed wound healing in defibrino-

generated animals. Buckman et al. (1977) found that short term post-operative defibrinogenation decreased breaking strength of sutured tendons. There are therefore some experimental evidence suggesting that the fibrin deposit in a wound plays a significant role in tissue repair. It seems, however, that these observations have not been subjected to controlled studies.

The purpose of the present investigation was

- to develop a model suitable to study the effect of defibrinogenation on fibrin deposition, wound healing and granulation tissue formation and allowing quantitative analysis, and
- to study the effect of defective fibrin deposition on
 - a) the cellular components of early granulation tissue,
 - b) wound strength development,
 - c) collagen accumulation in early granulation tissue, and
 - d) collagen synthesizing capacity of early granulation tissue.

MATERIAL AND METHODS

Experimental animals. The studies were performed on rabbits, weighing 2.3-4.0 kg. A total of 121 animals was used. The animals had free access to standard laboratory food and water.

Anesthesia. The animals were anesthetized by Veterinary Mebumal (ACO, Sweden, 30 mg/kg body weight) when necessary supplemented with open mask ether.

Experimental granulation tissue. The formation of granulation tissue was induced by subcutaneous implantation of viscose cellulose sponges (Kongfoss Fabrikker, Bygdø, Oslo) according to Viljanto & Kulonen (1962). Flat sponges were used, each weighing 60-65 mg (Pallin et al., 1975).

Defibrinogenation was achieved by injection of Arvin^R (Twyford Laboratories, London), 1 unit per kg body weight, into the marginal ear vein twice daily.

Experimental procedure. Each animal acted as its own control. Eight or 12 viscose cellulose sponges were implanted subcutaneously on the back of the rabbit through two standardized skin incisions which were closed by a continuous suture. Breaking strength of the skin incisions, and fibrin deposition and development of granulation tissue in the sponges, was studied after 4 or 7 days under control conditions using one side of the back. The breaking strength of the skin incisions was determined according to Sandblom et al. (1953) before removal of the sponges. The sponges were decapsulated and kept frozen until analysed.

The rabbits were allowed to recover for 14 days after sponge removal. Defibrinogenation was then started and was carried on for 3 days before new sponges were implanted on the other side of the back for an equivalent experimental period of 4 and 7 days. The defibrinogenation was continued throughout the experimental period.

In one study defibrinogenation was started on the second post-implantation day. The experimental setup was otherwise identical to the above described.

Blood was sampled using distal parts of the ear. Care was taken to prevent continuous bleeding after sampling.

Two ml of blood for determination of fibrinogen concentration and FXIII activity were taken into plastic tubes containing 0.5 ml of 3.8% trisodium citrate + 0.5 ml 1% AMCA. The tubes were immediately centrifuged and the plasma was kept frozen until studied.

Another 2 ml of blood for determination of FDP were collected in tubes containing 30 units of thrombin and 25 mg EACA. This blood was allowed to coagulate, serum was removed and was kept frozen until studied.

The hematocrit was determined in micro-hematocrit tubes.

The plasma fibrinogen concentration was determined according to Nilsson & Olow (1962).

Fibrin and fibrinogen degradation products in serum were determined with the immunochemical method described by Niléhn (1967) using a specific antiserum against rabbit fibrinogen. The specificity of the antiserum was tested by cross-immunoelectrophoresis.

Plasma factor XIII was assayed essentially as described by Lorand et al. (1972).

Hydroxyproline determination. Sponges were homogenized and the homogenate was hydrolyzed in HCl. Hydroxyproline in the hydrolysate was determined as described by Pikkarainen (1968).

Nucleic acids. DNA and RNA were extracted from the granulation tissue using the Schmidt-Thannhäuser procedure, as modified by Fleck & Munro (1962). RNA was estimated as ribose, as described by Ceriotti (1955). The results are given as RNA-ribose. DNA was determined by the diphenylamine reaction (Burton, 1956).

Enzyme activities. Sponges were homogenized in an ice bath. The homogenate was filtered through gauze and centrifuged. The supernatant was used for determination of enzyme activity. Acid and alkaline phosphatase activity was determined with the Biochemica Test-Combination^R kits, Boehringer, Mannheim. All enzyme determi-

nations were performed with at least two different dilutions of the homogenate.

Histology and enzyme histochemistry. Sponges used for microscopical examinations were either prepared by routine histological techniques, or immediately frozen and prepared under frozen conditions to demonstrate acid phosphatase activity according to Barka & Anderson (1962) and alkaline phosphatase activity as described by Aronsen et al. (1968).

Hemoglobin in granulation tissue was determined by the cyanmethemoglobin method (International Committee for Standardization in Haematology 1965).

^{14}C -proline incorporation in vitro. Tissue slices from two sponges were incubated in HEPES buffered Krebs Ringer solution in a thermostated shaking bath for 30 minutes, when 0.74 mBq Proline ($10\ \mu\text{l}$) $\text{L-}(^{14}\text{C}(\text{u}))$ (New England Nuclear, Boston, Mass.) was added to the solution and incubation was continued for $2\frac{1}{2}$ hours.

The cell activity was terminated by adding ethyl alcohol to the incubation mixture. The total radioactivity and the radioactivity of hydroxyproline was determined as described by Juva & Prockop (1966). Aqueous solutions were counted in a commercial scintillation mixture (Insta-Gel^R). Radioactivity was measured in a liquid scintillation counter (LKB Wallac 81000). At least 1 000 cpm over background were registered. ^{14}C -counting efficiencies were determined by external standardization using a standard quenching diagram prepared from samples obtained from granulation tissue with internal standardization (^{14}C -toluene).

Statistical methods. The means and SEM were calculated. The significance of the difference between the means was tested with Student

t-test for paired observations. Differences were considered significant when $p < 0.05$.

RESULTS

General effects of Arvin

Following Arvin administration *fibrinogen concentration in plasma* fell rapidly and remained at low to unmeasurably low levels for 10 days under continuous treatment. *Fibrinogen and fibrin degradation products (FDP)* were present during the whole period of defibrinogenation. High levels of FDP were observed during the first few days but declined later. Arvin administration was not followed by any demonstrable change in *FXIII activity*.

The animals did not lose weight and no untoward side effects were observed other than increased bleeding tendency at injection and sampling sites, especially during the first two days after the start of Arvin injection. There were no hematomas around the sponges. The *hematocrit* remained essentially unchanged.

Effects of defibrinogenation on early granulation tissue

Histological and histochemical appearance (I & III)

In sponges from the control period a well developed and preserved fibrin net was seen. In contrast, after defibrinogenation the fibrin net was irregular and the fibrin strands fragile and disrupted.

In both control and experimental sponges the dominating inflammatory cell was the polymorphonuclear leucocyte. The accumulation of PMN-leucocytes was more pronounced in sponges from the defibrinogenation period. The mononuclear cells in the inflammatory exu-

date were more sparsely represented and there was no marked difference between the two periods.

Defibrinogenation seemed to result in fewer fibroblast-like cells and less collagen in the sponges. Furthermore, the capillary ingrowth seemed delayed.

Histochemically alkaline phosphatase activity was mainly localized in the PMN-leucocytes. The intensity and the localization of the activity was not affected by defibrinogenation. The acid phosphatase activity was weak and localized to mononuclear cells, especially the macrophages.

Cell composition (III)

The total number of cells estimated by determination of DNA-content of the granulation tissue was not affected by defibrinogenation.

No difference of the RNA-content was demonstrable between the two periods. The RNA/DNA ratio was low indicating an immature tissue in both periods. Biochemical analyses of the sponge homogenates after defibrinogenation revealed increased activity of alkaline phosphatase indicating an accumulation of PMN-leucocytes. The acid phosphatase activity indicating the macrophage content was not different in the two periods.

The hemoglobin content of granulation tissue, indicating capillary ingrowth, was lower in the defibrinogenation period than in the control period.

Collagen accumulation and synthesis (II & IV)

On the fourth day the content of hydroxyproline in sponge induced granulation tissue was low. No difference was seen between the control and the defibrinogenation periods.

On the seventh day after implantation the granulation tissue from the defibrinogenation period had a lower content of hydroxyproline

than that from the control period. The total ^{14}C -proline activity as a measure of the protein synthesizing capacity and the ^{14}C -hydroxyproline activity as a measure of the collagen synthesizing capacity of the granulation tissue were reduced after defibrinogenation as compared to the control period. The ratio ^{14}C -hydroxyproline/ ^{14}C -proline was the same in both control and experimental periods.

Breaking strength of the skin incisions (II)

The breaking strength of the wounds was lower during defibrinogenation than in the control period both on the fourth and the seventh day.

Effect of delayed defibrinogenation on collagen accumulation in granulation tissue (V)

On the seventh day the collagen accumulation in granulation tissue was the same in the control and the defibrinogenated periods when defibrinogenation was started on the second postimplantation day.

DISCUSSION

When evaluating the significance of the initial fibrin formation for wound healing it seems logical to examine what happens in a wound deprived of this component. There are several agents which affect fibrin formation. Heparin inhibits clotting but is not suitable in a wound model since bleeding complications can be expected. Local or systemic application of fibrinolytic agents is difficult to control. In clinical practise defibrinogenating agents have been considered suitable for use in conjunction with surgery and their proteolytic action is specific and well studied (for review, see Stocker, 1978). Two of these, Batroxobin (Defib-

rase^R) and Ancrod (Arvin^R) are commercially available. Arvin was chosen since Defibrase in some studies has been reported to activate FXIII (the fibrin stabilizing factor) (Kopeć et al., 1969; Bell, 1974) leading to a consumption of this factor. Congenital deficiency of factor XIII has been associated with poor wound healing (Duckert, 1972).

Arvin is a proteolytic enzyme with a thrombin-like effect isolated from the venom of the Malayan pit viper, *Agkistrodon rhodostoma*, (Esnouf & Tunnah, 1967). When thrombin acts on fibrinogen two different peptides are released (fibrino-peptides A and B). The removal of both these peptides from fibrinogen is necessary for a polymerisation end-to-end and side-to-side resulting in the formation of a fibrin net which is then stabilized by FXIII. Arvin acts on fibrinogen by splitting off only fibrinopeptide A (Ewart et al., 1970; Bell et al., 1978) which results in an end-to-end polymerisation of the fibrin monomers. Factor XIII is not activated (Barlow et al., 1970; Pizzo et al., 1972). Arvin induced fibrin is not cross-linked and has poor mechanical properties and is more susceptible to digestion by plasmin (Kwaan & Barlow, 1971; Pizzo et al., 1972). Other coagulation factors than fibrinogen are not affected (Bell et al., 1968).

Arvin does not activate plasminogen *in vitro* (Pizzo et al., 1972) but a secondary recruitment of the fibrinolytic system is induced in the blood (Bell et al., 1978; Prentice et al., 1974). As a result of this activity fibrinogen-fibrin degradation products are observed in serum after Arvin administration (Vinazzer, 1971; Bell, 1973; Prentice et al., 1974). There is no significant effect on platelet count (Bell, 1973; Straub, 1973). A transient decrease of platelet factors III and IV has been recorded initially after start of defibrinogenation (Vinazzer, 1971). Antigene-

tic properties have been observed during repeated treatment (Pitney & Regoeczi, 1970; Vinazzer, 1973).

Thus, with intravenous infusions of Arvin a state of hypofibrinogenemia is achieved by converting plasma fibrinogen into an unstable form of fibrin which is then rapidly removed from the circulation.

The rabbit was chosen as experimental animal because its size allows repeated blood samples and repeated experiments which means that the same animal can be studied during a control and an experimental period. The wound healing process in this animal has also been thoroughly studied in previous investigations (Sandblom, 1944; Zederfeldt, 1957; Holmström, 1973).

A slight variation in effect between different batches of Arvin can be expected as they are obtained from different batches of venom. Therefore, in a separate series of experiments we tested the two batches used (I). Repeated injections of a standardized dose of Arvin did not interfere with the general condition of the animals as judged from weight development and general appearance. Increased bleeding tendency from injection and blood sampling sites was observed. To avoid bleeding the blood sampling frequency was reduced in the second series of experiments.

The plasma fibrinogen concentration fell rapidly after the first injections and could be kept below 1.0 g/l for at least 10 days which was sufficient for our purpose. The serum concentration of FDP was high during the first days of defibrinogenation but decreased later. This is consistent with previous reports (Bell, 1974 and 1978). As high concentration of FDP interferes with hemostasis (Olsson & Johnsson, 1972) one should start the defibrinogenation some days before the experiment. Therefore, sponge implantation was performed 3 days after the start of defibrinoge-

nation and no bleeding problems were encountered at the implantation sites. At this time one has also reached a stabilized situation with regard to fibrinogen and FDP concentration.

No change in FXIIIa activity was observed after Arvin administration.

Experimental granulation tissue induced by various subcutaneous implants has been used as a model for wound healing studies for a long time. For review see Viljanto, 1964. Viscose cellulose sponge was introduced as an inductive matrix by Viljanto & Kulonen (1962). The granulation tissue obtained in this way under normal conditions is very similar both histologically and chemically to that of healing wounds (Viljanto, 1964). The sponge model is suitable for quantitative biochemical analyses. However, the growth of connective tissue into an individual sponge equals the healing by secondary intention and the edge of collagen forming tissue advances gradually to the central parts of the sponge. This makes it difficult to separate distinctly the consecutive wound healing phases by analysing whole sponge content of early granulation tissue. However, this inconvenience can be reduced by the use of flat sponges (Pallin et al., 1975).

Viljanto & Kivikoski (1962) have histologically analysed the time sequences of granulation tissue formation in sponge implants. From these studies it is apparent that a dense fibrinous network develops within the pores of the sponges during the first 2 days. This is consistent with the histological appearance from the control sponges on day 4 and in the central parts of the sponges on day 7.

The fibrin deposited in sponges implanted in defibrinogenated animals does not form a dense regular pattern, instead it is sparse and scanty in histological appearance (I). Thus it is possible to

influence the fibrin deposit in a wound by systemic defibrinogenation. The main reason for the defective fibrin deposition in the sponges should be the low plasma fibrinogen concentration and the presence of FDP which are known to interfere with fibrin formation (Bang, 1962).

An inflammatory reaction precedes fibroplasia in healing wounds. The cellular components of this reaction has been studied by Leibovich & Ross (1975) and Kulonen (1976) among others. Their studies indicate clearly that the cellular response to trauma is of importance for fibroblast activation. Little is known about the non-cellular components and their role in fibroblast activation in granulation tissue. If defibrinogenation alters the inflammatory cellular reaction is not known. It is therefore essential to analyse the cell composition of granulation tissue formed during defibrinogenation.

The histological appearance of the granulation tissue harvested from the defibrinogenation period differed from that of the control period by the amount of inflammatory cells present (I & III). The dominating inflammatory cells, the PMN leucocytes, were more numerous after defibrinogenation. The mononuclear cells were in both periods more sparsely represented and no difference could be detected. It is difficult to make quantitative estimations from histological sections. The inflammatory cells in early granulation tissue, however, contain characteristic and measurable enzymes. Chemical determination of these can be used for quantitative evaluation. Alkaline phosphatase is mainly localized in PMN-leucocytes and mononuclear phagocytes, macrophages are characterized by their content of the lysosomal enzyme acid phosphatase (Carr, 1973). Histochemistry and biochemistry disclosed increased activity of alkaline phosphatase but no change in acid phosphatase acti-

vity after defibrinogenation. Thus, the biochemical findings are well correlated to the histological observation. The presence of an increased number of PMN-leucocytes after defibrinogenation in this study (III) indicates that defibrinogenation increases or prolongs the inflammatory cellular reaction. The reason for this remains to be elucidated.

The total number of cells, estimated by measuring the DNA content is not affected by defibrinogenation. Since the amount of PMN-leucocytes was found to be increased and no differences in macrophage content could be demonstrated it seems likely that the amount of fibroblasts is reduced in the defibrinogenation period.

Determination of wound breaking strength has commonly been used in experimental investigations of factors influencing wound healing (Sandblom, 1944; Zederfeldt, 1957). Determination of collagen deposition in granulation tissue also offers quantitative information about the healing process (Kulonen, 1970). Hydroxyproline is a for collagen almost unique amino acid (Neuman & Logan, 1950). Determination of hydroxyproline is thus a measure of collagen.

In this study (II) a comparison was made of the rate of healing of skin incisions and of granulation tissue under normal conditions as opposed to a defibrinogenated state. The model comprised two sets of wounds in the same animal with a time interval of 14 days. Sandblom & Muren (1954) have established that the rate of healing of wounds inflicted with short time interval in one animal is the same if the technique is kept uniform. Savlov & Dunphy (1954) have settled that there is no effect of one wound on another wound made at some other site of the body. Thus, in this model the defibrinogenation period could be compared with the control period.

On the seventh day of healing most of the tensile strength of a wound is due to its collagen content. The gain in strength of skin

wounds has been shown to correlate to the content of collagen developed in subcutaneously implanted sponges in the same animal during the first two weeks (Viljanto & Kulonen, 1962; Sandberg & Zederfeldt, 1963). As shown in the present investigation (II), there is a decrease in breaking strength of the skin incisions and in collagen content of granulation tissue on the seventh day after defibrinogenation. There is a good correlation between the decrease in breaking strength and in reduction of collagen deposition. The differences in breaking strength on day 4 between the control and the defibrinogenation periods probably reflect the different tensile properties of the clot induced by defibrinogenation, as there was no correlation to the collagen deposition of the granulation tissue at this time.

These results indicate that defibrinogenation with Arvin impairs wound strength development and the deposition of collagen in granulation tissue. Several studies have demonstrated or observed impaired wound healing in connection with defibrinogenation (Holt et al., 1970; Silberman & Kwaan, 1971; Olsson et al., 1973; Browse & Clemenson, 1978; Daniel et al., 1976).

Although hydroxyproline is an integral part of the polypeptide chains of extracellular, fibrillar collagen, hydroxyproline as such is never incorporated into collagen (Stetten, 1949). The hydroxyproline in collagen derives from an enzymic hydroxylation of a certain percentage of the proline residues incorporated in peptide linkages of the pro-collagen molecule (Prockop et al., 1973). These conditions make it possible to study the metabolism of collagen by using radioactively labelled proline as precursor and then determine specifically the radioactivity of hydroxyproline, which thus reflects the amount of newly synthesised collagen.

Tissue removed from the organism and placed in suitable buffers retains metabolic activity for many hours (Lampiaho & Kulonen, 1967; Niinikoski, 1969; Holmström et al., 1973). Radioactively labelled collagen precursors (^{14}C -proline) can be added to such a tissue culture. It is assumed that what happens *in vitro* reflects the synthetic capacity of the tissue at the time of harvesting. The *in vitro* method essentially measures the capacity to synthesize collagen under "ideal" conditions, that is the potential capacity of the enzyme systems, when the supply of oxygen and other substrates are not limiting. In a similar model Tengrup et al. (1980) have demonstrated a good correlation between *in vivo* and *in vitro* incorporation of ^{14}C -proline.

The capacity to synthesize collagen was found to be decreased in granulation tissue from the defibrinogenation period as compared to the control period (IV). The delay of collagen accumulation after defibrinogenation observed earlier (II) can well be explained by a decreased rate of synthesis.

It has been shown that blood supply is necessary for adequate fibroblast proliferation (Silver, 1973) and that lowered oxygen tension decreases collagen synthesis (Niinikoski, 1969). Evidence of delayed capillary ingrowth was found in the histological sections (I & III) and by the measurement of the Hb content of the granulation tissue (III).

In the defibrinogenation period not only collagen synthesis but also general protein synthesis (measured by incorporation of ^{14}C -proline into all proteins) was reduced. The ratio ^{14}C -hypro/ ^{14}C -proline was not changed by defibrinogenation. Since other cells in early granulation tissue (predominantly leucocytes) synthesize very little protein as compared to fibroblasts, the findings indicate that the reduced collagen synthesis after defibrinogenation

was due to a decreased number of active fibroblasts in the granulation tissue, which is corroborated by the findings in study III.

The impairing effect of Arvin on wound healing observed in this study can be attributed to its influence on the coagulum but also be the result of a direct effect of this agent on the fibroblast. Arvin does not have a lytic effect on preformed cross-linked fibrin *in vitro* (Pizzo et al., 1972) and should not significantly affect a preformed clot in this experimental model. Administration of Arvin starting after the deposition of a fibrin framework, which occurs in sponges predominantly during the first 2 days (Viljanto & Kivikoski, 1962) should thus offer a possibility to study an eventual effect of the substance directly on fibroblasts.

In such an experimental model (V) collagen accumulation is undisturbed.

Therefore the conclusion can be drawn that Arvin does not by direct action on fibroblasts interfere with the fibroblast function as estimated by the rate of collagen accumulation. The investigation (V) indirectly shows that the action of Arvin on wound healing is predominantly limited to the first phase of wound healing. Daniel et al. (1976) found that Arvin given only during the first two days postoperatively, caused a high incidence of wound dehiscence following endarterectomy in the dog. Further, Arvin added to the medium during incubation of granuloma slices with ^{14}C -proline does not affect collagen synthesis (Brändstedt, unpublished observations).

It seems clear from the present study that the impairment of the wound healing process after defibrinogenation with Arvin is not due to a direct effect of this agent on the fibroblast and therefore should be related to the changes in early fibrin deposition.

The mechanism by which defective fibrin deposition influences healing are not quite clear. Different possibilities can, however, be discussed.

Traditionally it has been thought that the fibrin strands act as basis and guidance to the advancing fibroblasts (Peacock & Van Winkle, 1976). This concept is based on visual criteria but supported by few experimental data. The main locomotory organ of the fibroblast when moving on a smooth surface, is its ruffled membrane. When moving on a substrate of fibers, the fibers seem to orientate the cell processes to a more elongated shape (Weiss, 1952 and 1961) suggesting that the cells move along these fibers. However, Stearns (1940) observing granulation tissue formation, on a day to day basis *in vivo* in transparent ear chambers, found no visual indication that fibrin was essential as guidance or support to the invading fibroblasts.

Whether or not the fibrin strands provide contact guidance for the migrating fibroblasts cannot be decided in the present investigation.

Some recent studies on fibronectin seem to provide a connection between fibrin and fibroblasts. On cultured fibroblasts fibronectin-fibrils are found running over and between cell surfaces (Ruoslahti et al., 1973).

Fibronectin binds to fibrin *in vitro* by both H^+ bonds and FXIII cross-linking and form complexes with fibrin *in vivo* (Sherman & Lee, 1979). It has been suggested that cross-linking of fibronectin to fibrin has an essential role in wound repair (Mosher, 1978). Since fibronectin is cross-linked to fibrin by FXIII, this would explain why some of FXIII deficient patients suffer from poor wound healing (Duckert, 1972).

The change in inflammatory cellular response observed can offer

an explanation to the delay in healing found after defibrinogenation. PMN-leucocytes are the first cells to appear in numbers in the area of a wound. These cells disappear after the first days of healing as normal repair progresses. There is a functional relationship between fibrin and the PMN-leucocytes in the early inflammatory exudate. Fibrin not only elicits a granulocyte cellular response, there is also morphologic evidence that the PMN-leucocytes are engaged in fibrin dissolution (Riddle & Barnhart, 1964). The present findings indicate that the presence of defect fibrin enhances or prolongs the PMN-leucocyte reaction. This is an indication of a changed inflammatory reaction which can in itself delay healing.

Studies during recent years have demonstrated growth promoting factors liberated from activated macrophages (Aalto et al., 1976; Aho & Kulonen, 1977) and from platelets (Kaplan et al., 1979). Platelet growth factor seems to be of importance specially for angiogenesis (Wall et al., 1978b). After defibrinogenation with Arvin there is delayed ingrowth both of fibroblasts and of capillaries. It seems, however, less likely that defects in growth stimulating factors should be responsible for these changes. Macrophages seem to be present in normal number and other studies (Bell, 1973 and 1974) have shown that Arvin does not affect platelets significantly.

The present study indicates that the early fibrin deposition in a wound is of importance for the subsequent repair process but does not allow firm conclusions as to the mechanisms involved.

CONCLUSIONS

The present investigation has shown that - systemic defibrinogenation using Arvin

- alters the fibrin deposit in the wound exudate and obviates the formation of a regular fibrin framework,
- changes the cellular response to a standardized trauma by increased or prolonged PMN-leucocyte cellular infiltration,
- delays the development of capillaries in granulation tissue,
- decreases the synthesis and the deposition of collagen in granulation tissue, and
- delays wound strength development in skin incisions.

Thus, this investigation indicates that the early fibrin deposition in a wound is of significance for the subsequent repair process.

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I

WOUND HEALING AND FORMATION OF GRANULATION TISSUE IN NORMAL
AND DEFIBRINOGENATED RABBITS

AN EXPERIMENTAL MODEL AND HISTOLOGICAL STUDY

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Wound Healing and Formation of Granulation Tissue in Normal and Defibrinogenated Rabbits

An Experimental Model and Histological Study

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Key Words. Arvin® · Granulation tissue · Fibrin · Wound healing

Abstract. The aim of this investigation was to develop an experimental model for studies on the significance of fibrin deposition for wound healing. Systemic defibrinogenation with Arvin® was used to achieve abnormal fibrin deposition in subcutaneously implanted cellulose sponges. The formation of granulation tissue in the sponges was studied histologically. Arvin injections resulted in a rapid decrease in plasma fibrinogen concentration with a concomitant rise in fibrin-degradation products. No change in FXIII_a activity was seen. During defibrinogenation an abnormal fibrin deposition was found in the sponges. The fibrin strands appeared irregular and disrupted. The number of fibroblasts and collagen fibrils was reduced in granulation tissue formed during defibrinogenation. The model used seems to allow controlled studies on the significance of fibrin deposition for wound healing. Defibrinogenation was found to influence granulation tissue formation.

The fibrin net formed in a wound appears to act as a scaffold for migrating fibroblasts (18). *Fleisher and Loeb* (5) suggested that fibrin formation and resolution were important factors in the regulation of tissue repair. Several recent studies support this hypothesis: delayed wound healing has been demonstrated in defibrinogenated animals (7, 15, 19) and in animals lacking FXIII (the fibrin-stabilizing factor) (20). Increased formation of connective tissue has been found after inhibition of fibrinolysis (8). Other studies (9) have, however, failed to demonstrate any effect of defibrinogenation on wound strength.

The aim of this study was to develop an experimental model to study the significance of fibrin deposition for granulation tissue formation.

Material and Methods

27 rabbits of both sexes, weighing between 2.3 and 4.0 kg, were used. The animals were kept in separate cages and given food pellets (Astra-Ewos, Sweden) and water *ad libitum*.

Arvin® (Batch G 1597 Laboratory grade, supplied by Twyford Laboratories, London) was used for defibrinogenation. Viscose cellulose sponge material (Kongfoss fabrikker, Bygdø, Oslo) was used to induce formation of granulation tissue.

7 rabbits (group I) were used to study the effect of a standardized dose of Arvin on plasma fibrinogen. In addition, the concentration of fibrin-degradation products (FDP) in serum was determined and in 2 of the rabbits also FXIII_a activity. 20 rabbits (group II) were used to study fibrin deposition and granulation tissue development in viscose cellulose sponges under control and defibrinogenated conditions.

Group I

Arvin was given intravenously in a dose of 1 unit/kg body weight twice daily. Blood samples for determination of plasma fibrinogen concentration, FXIII_a activity and FDP concentration in serum were taken daily for 4 days and then every other day to day 10.

2 ml of blood was taken from the marginal ear vein into plastic tubes containing 0.5 ml of 3.8% trisodium citrate. The tubes were immediately centrifuged for 20 min at 2,000 g and the plasma frozen at -20 °C for later determination of fibrinogen concentration and FXIII activity. Another 2 ml was collected in tubes containing 30 units of thrombin and 25 mg EACA. This blood was allowed to stand for 2 h at room temperature to coagulate, serum was removed and frozen at -20 °C for later determination of FDP.

The hematocrit was determined in micro-hematocrit tubes centrifuged at 10,000 g for 10 min.

The plasma fibrinogen concentration was determined according to Nilsson and Olow (12). Corrections were made for the dilution of plasma by citrate according to Gram's formula.

Fibrin and fibrinogen degradation products in serum were determined with the immunochemical method described by Nilén (11) using a specific antiserum against rabbit fibrinogen. The specificity of the antiserum was tested by cross-immunoelectrophoresis.

Plasma factor XIII was assayed essentially as described by Lorand *et al.* (10), determining incorporation of ¹⁴C-putrescine into casein catalyzed by FXIII. The following modification were made. After addition of 5 µl of 50% aqueous glycerol and 10 µl of 25 mM dithiothreitol to 20 µl of plasma the mixture was kept at 56 °C for 4 min. After cooling to 37 °C bovine thrombin (Topostasin Roche; 10 µl of 100 IU/ml saline) was added. After 20 min at 37 °C, Hammersten casein (Merck; 30 µl 2.2%) and ¹⁴C-putrescine (Amersham CFA 301 Batch 28; 10 µl of 3.0 mM specific activity 27.8 Ci/mol) were added. The reaction was then initiated by the addition of calcium chloride (10 µl of 40 mM). If not otherwise specified, 50 mM Tris chloride buffer, pH 7.5 was used as solvent.

Termination of the reaction and removal of incorporated labelled ¹⁴C-putrescine were carried out as described by Lorand *et al.* (10). Samples of 5 µl were taken 20 min after initiating the reaction. The radioactivity was measured in a liquid scintillation counter (LKB Wallac 81000).

Group II

These animals were shaved on one side of the back and anesthetized with Veterinary Mebumal® (ACO, Sweden) supplemented with open mask ether. Under sterile conditions 12 cellulose sponges were implanted subcutaneously according to *Viljanto and Kulonen* (23) as modified by *Pallin et al.* (17).

After a period of 4 (6 rabbits) or 7 (14 rabbits) days all sponges were removed. Two sponges were used for histological examination. After this control period the rabbits were allowed to recover for 14 days and defibrinogenation was then started according to the schedule used in group I.

On the 4th day of defibrinogenation, sponges were implanted, as in the control period using the other side of the back, and left in place for 4 and 7 days, respectively. The defibrinogenation was continued throughout the experimental period.

Plasma fibrinogen concentration was determined on the day of sponge implantation and removal.

After removal the cellulose sponges were immediately fixed in 10% neutral formalin, serially sectioned at 4 μ m and stained according to van Gieson.

Results

General Conditions. The animals appeared well and did not loose weight. Increased bleeding tendency was observed at injection sites during the first days of Arvin treatment. There were no hematomas around the sponges. The hematocrit remained essentially unchanged.

Group I

Fibrinogen Concentration in Plasma. The fibrinogen concentration fell rapidly after the first Arvin injection and was then constantly below 1.0 g/l (fig. 1).

Fibrinogen Degradation Products in Serum. FDP were present during the whole period and high levels were observed during the first days of Arvin treatment (fig. 1).

FXIII in Plasma. No decrease in platelet-poor plasma FXIII_a activity was observed during defibrinogenation (fig. 2).

Group II

The plasma fibrinogen concentration, at the time of implantation and removal of the sponges, varied between immeasurably low and 0.6 g/l.

Wound Healing in Defibrinogenated Rabbits

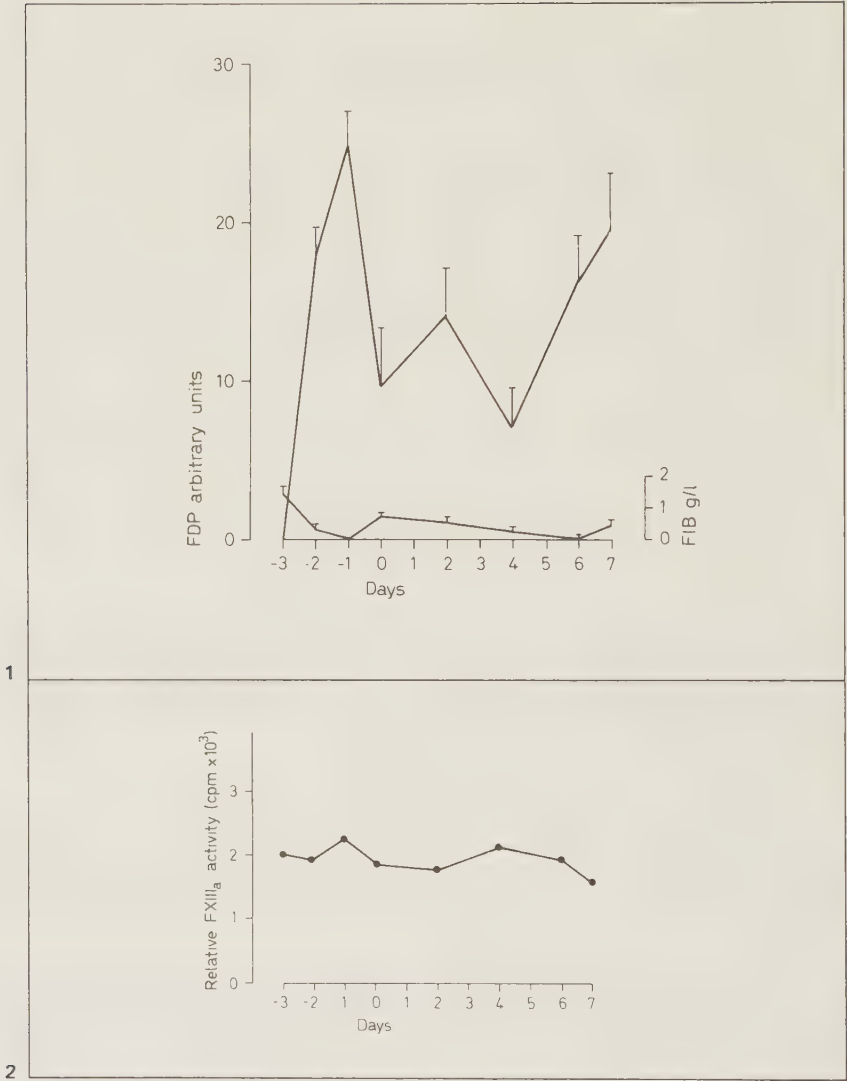


Fig. 1. FDP and fibrinogen concentration in Arvin-treated rabbits. Both curves represent mean ($\pm$ SEM) obtained on blood samples from 7 rabbits (group I). FDP was measured with electroimmunoassay. Three fractions of FDP were observed and all behaved in a similar way. Only the most prominent fraction is recorded.

Fig. 2. FXIII concentration in Arvin-treated rabbits. FXIII values of platelet-poor plasma samples obtained from 2 animals as measured by incorporation of ^{14}C -putrescine into casein catalyzed by FXIII_a.

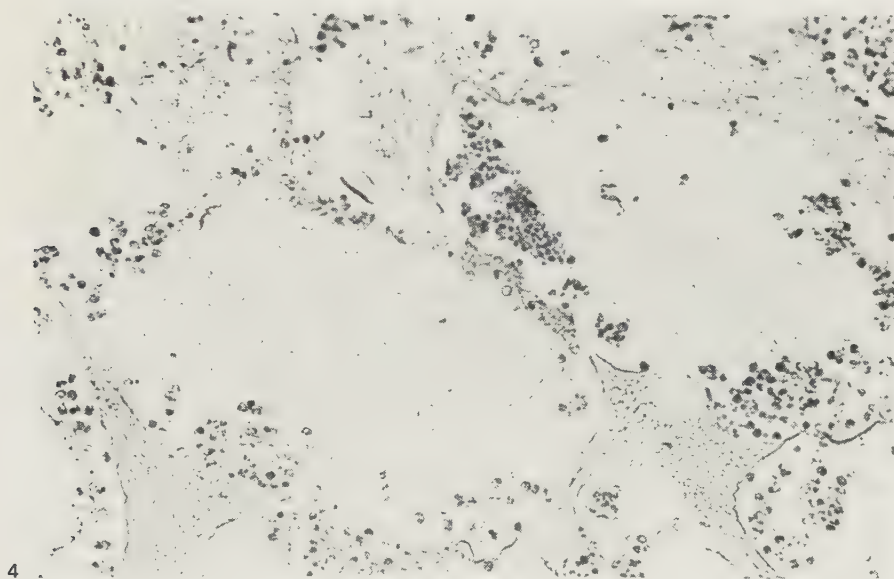


Fig. 3. 4th day, control. Margin of sponge with fibrin network and scattered inflammatory cells. van Gieson. $\times 320$.

Fig. 4. 4th day, defibrinogenation. Pronounced inflammatory exudate with leucocytes and a disrupted fibrin network with granulated fibrin strands. van Gieson. $\times 320$.

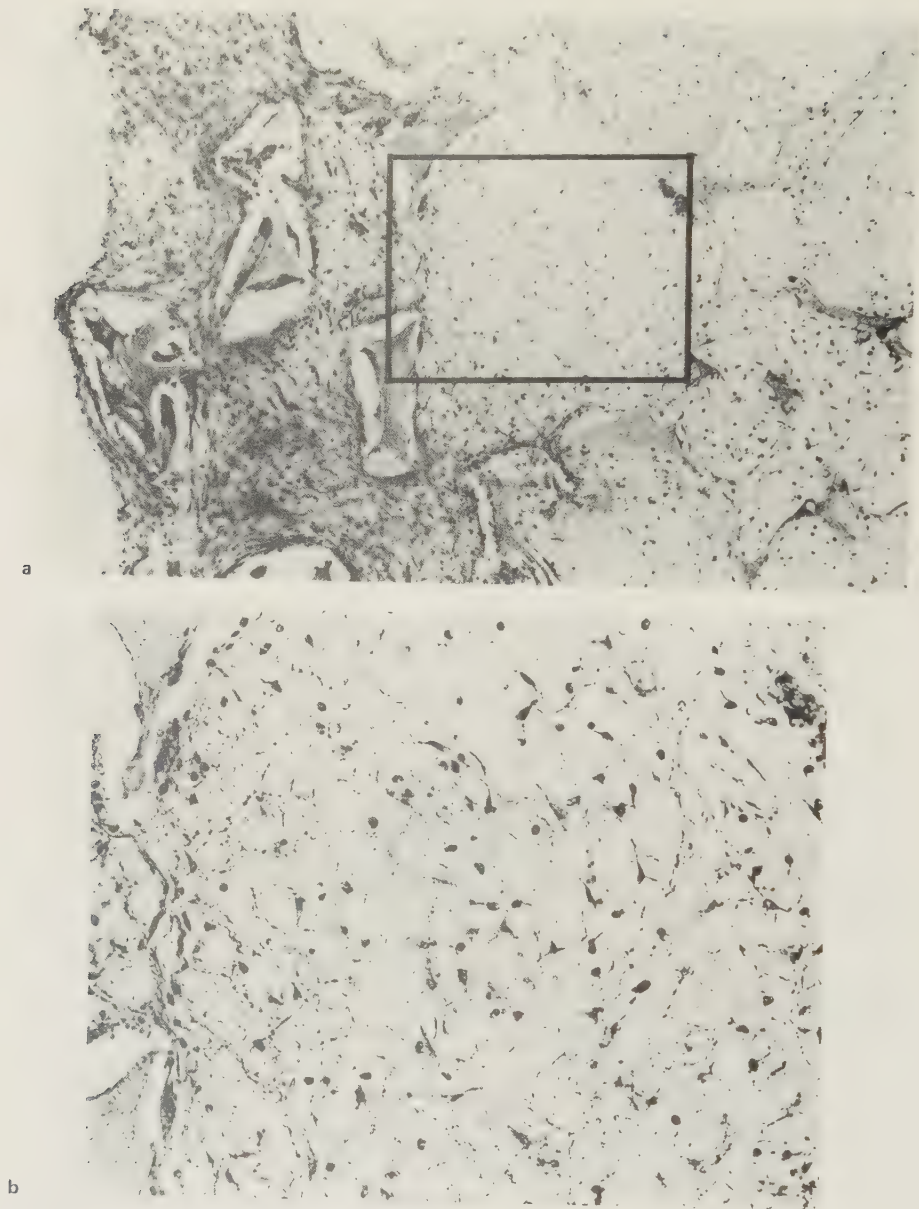


Fig. 5. 7th day, control. Granulation tissue with capillary blood vessels and a well-preserved fibrin net and elongated fibroblasts. van Gieson. **a** $\times 125$. **b** $\times 320$.

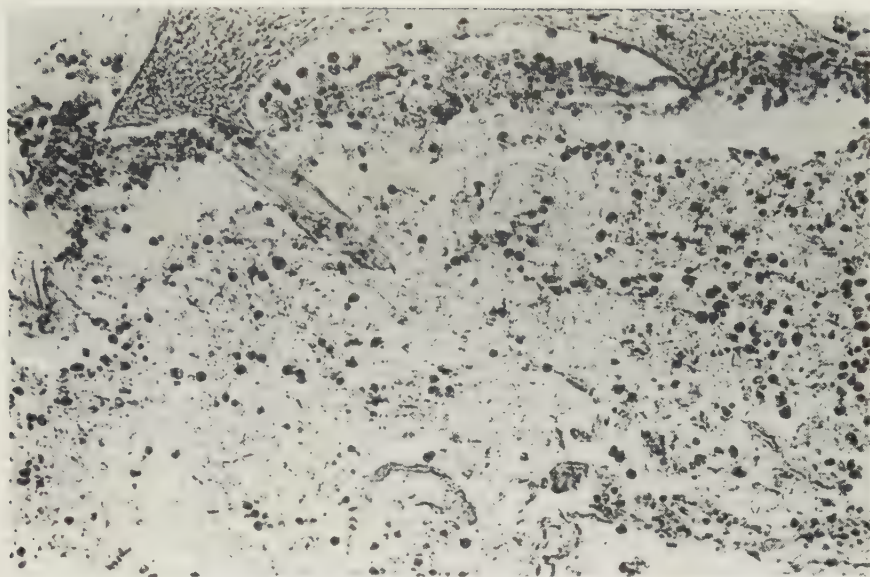


Fig. 6. 7th day, defibrinogenation. Impaired fibroplasia and damaged fibrin network. van Gieson. $\times 320$.

Histological Findings

On the 4th day an inflammatory exudate of fibrin and polymorphonuclear leucocytes was observed. Peripherally there were small foci of fibroblasts and occasional collagen fibrils.

In the sponges from the control period the fibrin strands were well-preserved and arranged in a network throughout the sponges (fig. 3). After defibrinogenation a fibrin network was present but appeared fragile, irregular and disrupted (fig. 4). Furthermore, the leucocyte accumulation appeared somewhat more pronounced in these sponges.

On the 7th day areas of granulation tissue with fibroplasia and newly formed capillary blood vessels were seen at the periphery of the sponges. In the same area macrophages appeared to be in connection with the sponge material.

In the sponges from the control period the zone of fibroplasia at the periphery surrounds a zone of elongated fibroblasts distributed in a fibrin net. Further towards the center there were partly disintegrated leucocytes (fig. 5a, b). After defibrinogenation the zone of peripheral granulation tissue was less marked than that from the control period. The number of fibroblasts and the amount of

collagen fibrils appeared less. The fibrin network was scanty and the fibroblasts were arranged in a disorderly manner (fig. 6). The leucocyte accumulation seemed slightly increased.

Discussion

The present study was performed to evaluate the significance of fibrin for granulation tissue development.

To achieve abnormal fibrin deposition we used a purified fraction of the Malayan pit viper venom, Arvin, with a thrombin-like effect on the coagulation system (4, 13, 21), which produces hypofibrinogenemia by converting plasma fibrinogen which aggregates to microclots which are rapidly removed from the circulation. A secondary fibrinolysis takes place. The appearance of FDP is a consequence of the fibrinolysis. Following Arvin injections a marked decrease of plasma fibrinogen levels was found during the whole period studied.

No apparent effects of Arvin on other blood coagulation factors have been reported with the exception of a possible influence on FXIII (3, 21, 24). Since some wound-healing disturbances have been associated with a depletion of FXIII - for review see *Folk and Finlayson* (6) - it seemed important to check that the batch of Arvin used for defibrinogenation in these experiments did not deplete factor XIII activity. No loss of activity could be demonstrated in the 2 animals studied.

During the control period a well-developed and preserved fibrin net was found in the sponges both 4 and 7 days after implantation. In contrast the fibrin net was irregular and the fibrin strands fragile and disrupted in the sponges implanted during defibrinogenation.

The defective fibrin deposition in the sponges can be explained by the low plasma fibrinogen concentration and possibly by the presence of FDP which are known to interfere with fibrin formation (2).

During pilot studies we encountered some difficulties with bleeding from the sampling sites - the marginal ear vein. FDP is reported to increase bleeding tendency by its anticoagulant effect (16). When surgery was performed after the initial high rise of FDP there were no problems with bleeding. To avoid disturbances from bleeding, sampling was kept to a minimum in the experimental group (II). The effect of a standardized dose of Arvin was tested separately (group I). As *Ashby et al.* (1), we observed that animals operated on responded more to a given dose of Arvin than nonoperated animals.

Sponge-induced granulation tissue obtained under normal conditions is both histologically and chemically very similar to that of healing wounds (22).

There was a distinct difference between the histological appearance of the granulation tissue in sponges from the control period and that from the defibrinogenation period. It is apparent in the histological sections from day 7 that defibrinogenation results in a less mature granulation tissue as indicated by lack of ingrowth of fibroblast-like cells and absence of collagen deposition.

The findings are consistent with the histological observations of others (7, 19) in different experimental models. *Olsen and Pitney* (14) found an inhibition of fibroblast formation in organizing pulmonary emboli in rabbits made hypofibrinogenemic by Arvin. *Olsson et al.* (15) observed impaired wound healing in defibrinogenated dogs used for vein graft surgery.

The present study indicates that normal fibrin deposition is of significance for granulation tissue formation. The described model offers possibilities for further studies of the mechanisms involved.

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II

EFFECT OF DEFIBRINOGENATION ON WOUND STRENGTH AND COLLAGEN FORMATION

A STUDY IN THE RABBIT

S. BRÄNDSTEDT AND P.S. OLSON

ABSTRACT

Wound healing was studied in Arvin^R-defibrinogenated rabbits by determinations of skin wound strength and by analysis of collagen content in sponge induced granulation tissue. Strength development was found to be delayed in skin wounds as was the accumulation of collagen in granulation tissue. These results indicate that the initial fibrin formation in a wound is of significance for repair.

Defibrinogenation is used therapeutically in thromboembolic disorders (Bell, 1974; Lowe et al., 1978) and in peripheral arterial disease (Tønnesen et al., 1978; Ehrly, 1978); its use in conjunction with vein surgery has been discussed (Olsson et al., 1973; Browse & Clemensson, 1978).

In a previous investigation (Brändstedt et al., 1980) an experimental model to study the significance of fibrin formation for wound healing was described. In defibrinogenated animals granulation tissue formation in sponge implants was histologically found to be impaired and collagen deposition seemed reduced. Other studies using different models (Holt et al., 1970; Silberman & Kwaan, 1971) have reported similar findings. The aim of the present investigation is to establish if defibrinogenation influences strength development in wounds and if the impairment of collagen deposition observed histologically can be verified by quantitative biochemical methods.

MATERIAL

Thirty-five rabbits of both sexes, weighing between 2.3 and 4.0 kg were used. The animals were kept in separate cages and given food pellets (Astra-Ewos, Sweden) and tap water *ad libitum*.

Arvin^R (Laboratory grade, batch G 1597, Twyford Laboratories, London) was used for defibrinogenation.

Viscose cellulose sponge material (Kongfoss Fabrikker, Bygdø, Oslo, Norway) was used to induce granulation tissue formation.

METHODS

Experimental procedure

The experimental procedure is schematically illustrated in Fig. 1. The animals were anesthetized by Veterinary Mebumal (ACO, Sweden, 30 mg/kg body weight) and shaved on one side of the back. Through two standardized incisions in the shaved area 12 cellulose sponges, each weighing 60-65 mg, were implanted subcutaneously under

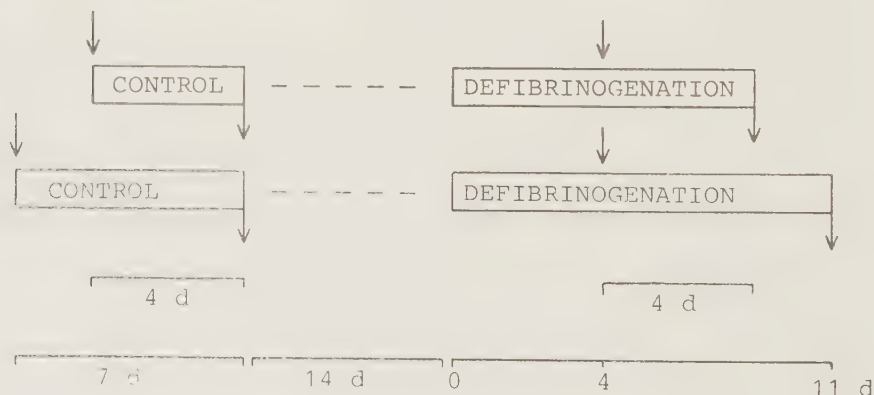


Fig. 1. Boxes indicate experimental and control periods and arrows indicate time of sponge implantation and removal.

sterile conditions as described by Viljanto & Kulonen (1962). The wounds were closed by 3-0 Ethilon^R with stitches 5 mm apart. After a control period of 4 or 7 days, the sponges were removed for analysis of their hydroxyproline content. The breaking strength of the skin incisions was determined in 13 rabbits according to Sandblom et al. (1953). Two determinations were made on each wound. The wounds were resutured.

After a recovery period of 14 days defibrinogenation was started with Arvin^R (1 unit per kg body weight) given twice daily in the marginal ear vein as described previously (Brändstedt et al., 1980). On the fourth day of defibrinogenation wounds were inflicted and sponges implanted on the other side of the back exactly as in the control period. The breaking strength was determined and the sponges removed after 4 or 7 days as was done in the control period. The defibrinogenation was continued throughout the experimental period. Determinations of fibrinogen concentration in plasma were made at the time of sponge implantation and removal.

Hydroxyproline determination

The carefully decapsulated sponges were homogenized in distilled water with an Ultra Turrax^R homogenizer for 30 sec. The homogenate was hydrolyzed in 6 N hydrochloric acid for 3 hours at 130^o C. The hydrochloric acid was removed by evaporation in a water bath. Hydroxyproline in the hydrolysate was determined as described by Pikkarainen (1968).

Plasma fibrinogen concentration

The plasma fibrinogen concentration was determined according to Nilsson & Olow (1962).

Statistical calculations

Student t-test was used for testing the significance of differences between paired observations.

RESULTS

The plasma fibrinogen concentration after defibrinogenation measured at the time of sponge implantation and removal, ranged from immeasurably low to 0.6 g/l.

Breaking strength of skin incisions. The breaking strength of the wounds was lower during defibrinogenation than in the control period both on the fourth and the seventh day (Table I, Fig. 2).

TABLE I. Breaking strength of skin incisions on rabbits obtained from Arvin^R-defibrinogenated and control periods.

Days postop	No of animals	Control BS newton	Arvin BS newton
		M $\pm$ SEM	M $\pm$ SEM
4	6	1.1 $\pm$ 0.2	0.3 $\pm$ 0.1 *
7	7	3.7 $\pm$ 0.4	2.3 $\pm$ 0.4 *

*P<0.01

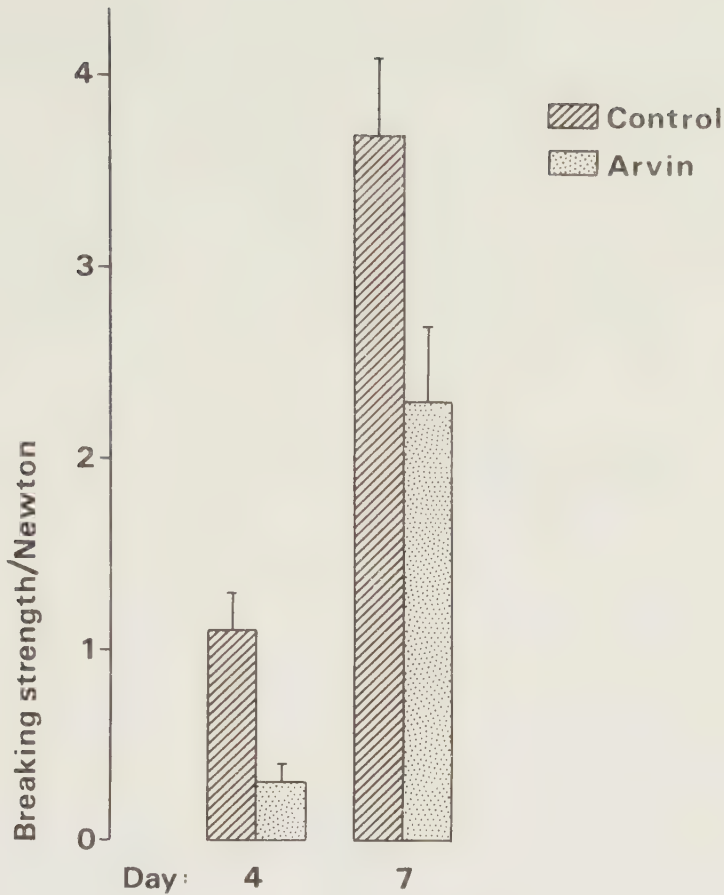


Fig. 2. Breaking strength of skin wounds. Height of columns indicate mean load at time of disruption. $\pm$ SEM.

Hydroxyproline (Table II, Fig. 3). The content of hydroxyproline in sponge induced granulation tissue was low on the fourth day. No difference was seen between the control and the defibrinogenation period. On the seventh day the granulation tissue from the defibrinogenation period had a lower content of hydroxyproline than that from the control period.

TABLE II. Hydroxyproline content in sponge induced granulation tissue expressed in μg per granuloma from Arvin^R defibrinogenated and control periods.

Days postop	No animals	Control Hypro		Arvin Hypro	
		M	$\pm$ SEM	M	$\pm$ SEM
4	6	41	$\pm$ 4	44	$\pm$ 5
7	29	54	$\pm$ 5	40	$\pm$ 4*

* $P < 0.001$

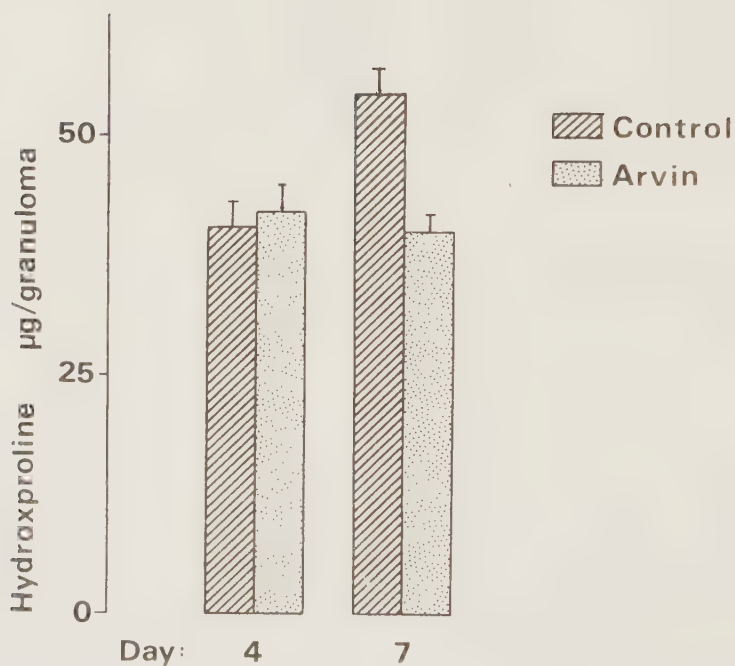


Fig. 3. Hydroxyproline content in sponge induced granulation tissue. Height of columns indicate mean content. $\pm$ SEM.

DISCUSSION

The development of wound strength in healing incisions is functionally important, and determinations of wound strength are commonly used in experimental investigations as a quantitative test of the rate of repair (Sandblom, 1944; Zederfeldt, 1957). Determination of collagen deposition in granulation tissue gives further information about the healing process (Kulonen, 1970).

In this study a comparison was made of the rate of healing under normal conditions and during a state of induced hypofibrinogenemia in the same animal. The model comprises two sets of wounds in the same animals with a time interval of 14 days. The wounds and sponges were symmetrically positioned, and the experimental technique strictly standardized.

Sandblom & Muren (1954) studied the rate of healing of wounds inflicted with short time interval in the same animal and established that if the technique is kept strictly the same, there is no difference in the rate of healing between the first and the second set of wounds. Savlov & Dunphy (1954) have settled that there is no effect of one wound on another wound made at some other site of the body. Consequently in this experimental model, any differences in wound healing during the control and the experimental period should be an effect of the defibrinogenation by Arvin^R.

The cellulose sponge model is useful to obtain granulation tissue for quantitative studies (see Kulonen). However, there is a time difference in the development of tensile strength of skin wounds and that of granulation tissue formed in implanted sponges (Viljanto, 1964; Niinikoski, 1969) which must be kept in mind when interpreting the results. The temporal difference between

the two models means that the granulation tissue of the wounds is more mature than the tissue that has grown into the sponges in the same time period.

In the present investigation the breaking strength of skin incisions was found to be decreased during defibrinogenation with Arvin^R, as compared to controls both on the fourth and seventh day, whereas the content of the granulation tissue from the sponges showed a difference only on the seventh day.

During the lag phase of healing, the principal contributor to breaking strength is the fibrin in the wound. The difference in breaking strength on the fourth day is likely to be a reflection mainly of the physical properties of the clot. The quality of the clot is changed by defibrinogenation. The changes can be due to decreased amount of fibrin, but it is also known that fibrin degradation products (FDP) which appear after defibrinogenation interfere with fibrin polymerization (Hirsch et al., 1965; Regoeczi, 1971). On the seventh day of healing most of the tensile strength of a wound is due to its collagen content. The gain in strength in skin wounds has been shown to correlate to the content of collagen developed in subcutaneously implanted sponges in the same animal during the first two weeks (Sandberg & Zederfeldt, 1963; Viljanto & Kulonen, 1962). As shown in this investigation, there is a difference in collagen content of granulation tissue on the seventh day. The differences in breaking strength of the skin incisions in this study correlate well with the differences in the collagen content of the granulation tissue on day seven.

These results indicate that defibrinogenation with Arvin^R impairs wound healing and deposition of collagen in granulation

tissue. Several studies have demonstrated or observed impaired wound healing in connection with defibrinogenation (Silberman & Kwaan, 1971; Holt et al., 1970; Olsson, P et al., 1973; Browse & Clemenson, 1978; Daniel et al., 1976). In a previous study (Brändstedt et al., 1980), it could be demonstrated that Arvin^R changes the fibrin framework developed in wound exudate within implanted sponges. Fleischer & Loeb (1915) have postulated that fibrin formation and resolution are important factors in the regulation of normal tissue repair. Our studies fit into this concept as do other investigations (Kwaan & Astrup, 1969).

It thus seems likely that the observed effects of Arvin on wound healing are due to its defibrinogenating properties. Definite conclusion requires, however, that possible effect of Arvin on other factors of importance for healing are also evaluated.

The effect of Arvin on fibroblast function and on inflammatory reaction will therefore be studied.

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III

CELL COMPOSITION OF GRANULATION TISSUE IN DEFIBRINOGENATED RABBITS

S. BRÄNDSTEDT, F. RANK, P.S. OLSON AND J. AHONEN

CELL COMPOSITION OF GRANULATION TISSUE IN DEFIBRINOGENATED RABBITS

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(Submitted for publication February 20, 1980)

Abstract. The influence of systemic defibrinogenation on cell composition and capillary formation in sponge-induced early granulation tissue was studied in rabbits. The tissue was studied histologically and the content of RNA, DNA and Hb was determined. Inflammatory cells were identified and quantified using histochemical and biochemical techniques. An accumulation of PMN-leucocytes was found after defibrinogenation whereas the amount of fibroblasts and capillaries was reduced.

The initial inflammatory cellular reaction in wounds is of importance for wound debridement and the subsequent activation of fibroblasts (Leibovich & Ross, 1975), which results in the formation of collagen.

Systemic defibrinogenation interferes with the deposition of fibrin in wound exudate (Brändstedt, Olson & Rank, 1980). Delay in wound strength development (Holt, Holloway, Raghupati & Calnan, 1970; Silberman & Kwaan, 1971; Olsson, Ljungqvist & Göransson, 1973; Daniel, Pizzo & McKee, 1976; Browse & Clemenson, 1978; Brändstedt & Olson, 1980) and in accumulation of collagen in granulation tissue (Holt et al.; Brändstedt et al.) has been demonstrated after defibrinogenation.

If defibrinogenation alters the inflammatory cellular reaction is not known. The aim of the present study was to analyse the cell composition and the vascularity of granulation tissue formed during defibrinogenation.

MATERIAL

Twenty-seven rabbits of both sexes, weighing between 2.3 and 4.0 kg, were used. The animals were kept in separate cages and given food pellets (Astra-Ewos, Sweden) and water ad libitum.

Arvin® (Batch G 1597 Laboratory grade, supplied by Twyford Laboratories, London) was used for defibrinogenation.

Viscose cellulose sponge material (Kongfoss fabrikker, Bygdø, Oslo) was used to induce formation of granulation tissue.

METHODS

Experimental procedure

The experimental model has been described previously in greater detail (Brändstedt et al.). Each animal acted as its own control. The animals were anesthetized with Veterinary Mebumal® (ACO, Sweden, 30 mg/kg body weight) and shaved on one side of the back. Under sterile conditions two skin incisions were made in the shaved area. Through each wound six sponges (each weighing 60–65 mg) were implanted subcutaneously as described by Viljanto & Kulonen (1962). The sponges were removed after seven days. The wounds were resutured.

After a recovery period of fourteen days defibrinogenation was started with Arvin® (1 unit/kg body weight) given twice daily in the marginal ear vein. On the fourth day of defibrinogenation sponges were implanted on the other side of the back exactly as in the control period. The sponges were removed for analysis after seven days as was done in the control period. Defibrinogenation was continued throughout the experimental period.

Plasma fibrinogen concentration was determined on the days of sponge implantation and removal according to Nilsson & Olow (1962).

The sponges were carefully decapsulated before processing.

Nucleic acids. DNA and RNA were extracted from the granulation tissue using the Schmidt-Thannhäuser procedure, as modified by Fleck & Munro (1962). RNA was

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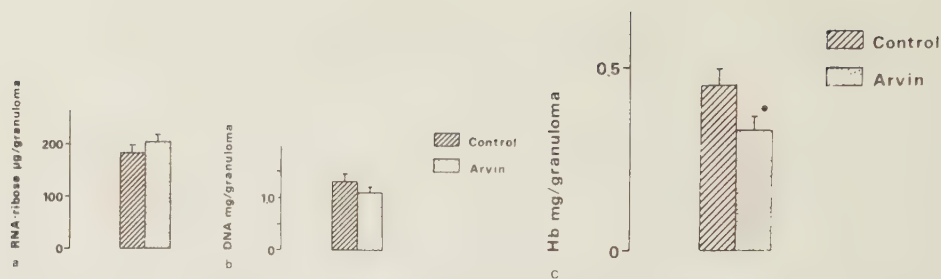


Fig. 1. Effect of defibrinogenation (compared to control period) on chemical components of 7-day-old experimental granulation tissue. (a) DNA, (b) RNA-ribose, (c) tissue

haemoglobin. Mean $\pm$ SEM. Significant difference between the groups is indicated ($p < 0.05$).

estimated as ribose, as described by Ceriotti (1955). D-ribose (A-grade, Lot 800003, Calbiochem, San Diego, Calif.) was used as standard. The results are given as RNA-ribose. DNA was determined by the diphenylamine reaction (Burton, 1956). Sodium salt of salmon sperm deoxy-ribose-nucleic acid (A-grade, highly polymerized, Lot 410141, Calbiochem, San Diego, Calif.) was used as standard.

Enzyme activities. The sponges were homogenized in ice cold 0.9% sodium chloride with an Ultra-Turrax® homogenizer for 2×7.5 sec in an ice bath. The homogenate was filtered through gauze and centrifuged twice at 600 g for 15 min at 4°C . The supernatant was used for determination of enzyme activity. Acid and alkaline phosphatase activity was determined with the Biochemica Test-Combination® kits, Boehringer, Mannheim as specified in the kit manuals. All enzyme determinations were performed with at least two different dilutions of the homogenate.

Histology and enzyme histochemistry. One sponge from each animal was prepared using routine histological techniques and sections of four microns were stained with haematoxylin-eosin and van Gieson. Another sponge was immediately frozen in propane cooled by liquid nitrogen in a container. After storage at -70°C , the sponges were

transferred to a cryostat and sections about six microns were processed to demonstrate acid phosphatase activity according to Barka & Anderson (1963) and alkaline phosphatase activity as described by Aronsen, Hägerstrand & Nordén (1968).

Haemoglobin in granulation tissue was determined in one sponge from each wound. The sponge was homogenized in 10 ml of water. The homogenate was centrifuged at 20 000 g for 30 min at $+4^{\circ}\text{C}$. The haemoglobin of the supernatant was determined by the cyanmethaemoglobin method (International Committee for Standardization in Haematology 1965).

Statistical calculations. Student *t*-test for paired observations was used for the statistical evaluation of the differences of the means.

RESULTS

Fibrinogen concentration in plasma at the time of implantation and removal of the sponges varied between immeasurably low to at the most 0.6 g/l.

Haemoglobin content of granulation tissue from the defibrinogenation period was significantly lower than from the control period (Fig. 1).

DNA content showed no statistically significant difference between the two periods (Fig. 1). No

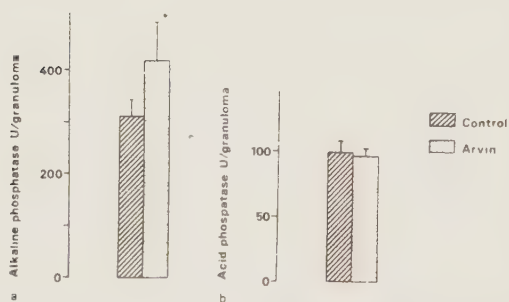


Fig. 2. Effect of defibrinogenation (compared to control period) on enzyme activities of 7-day-old experimental granulation tissue. (a) alkaline phosphatase activity. (b) Acid phosphatase activity. Mean $\pm$ SEM. Significant difference between the groups is indicated ($p < 0.05$).

Table 1. Quotients of mean values of RNA to DNA and of alkaline and acid phosphatase activity to DNA

Quotients	Control	Arvin
RNA/ DNA	0.12	0.16
Alkaline phosphatase/ DNA	0.29	0.47
Acid phosphatase/ DNA	0.09	0.11

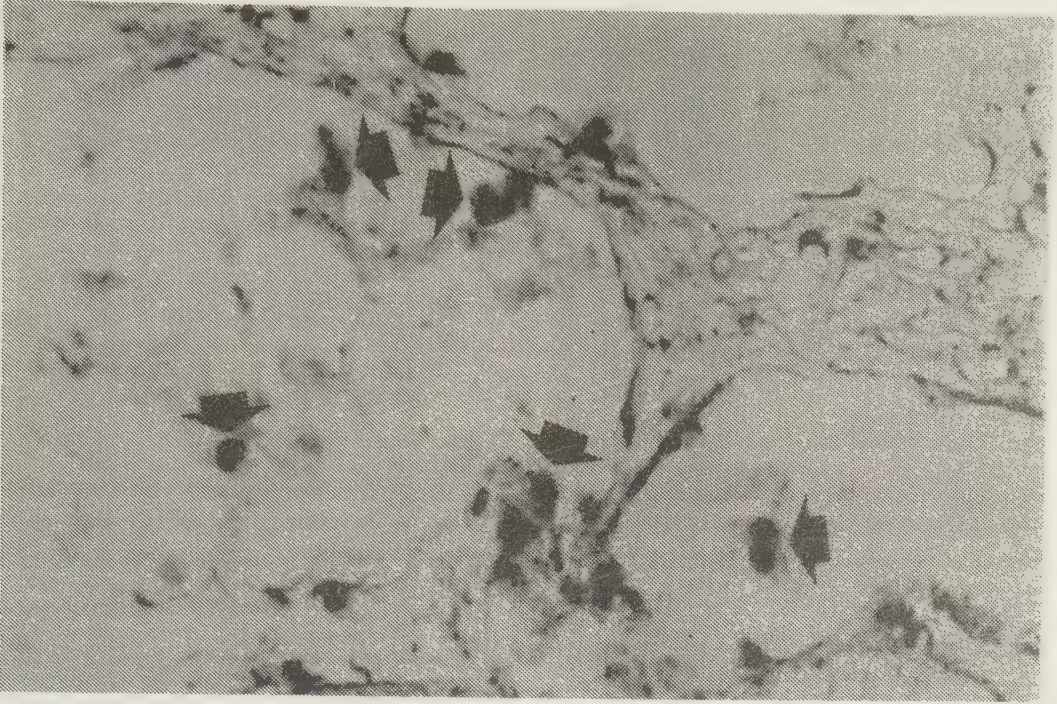


Fig. 3. PMN-leucocytes displaying alkaline phosphatase activity ($\times 400$).

difference of the RNA content was demonstrable between the two periods (Fig. 1).

Enzyme activities. The alkaline phosphatase activity in the granulation tissue determined biochemically was significantly higher in granulation tissue from the defibrinogenation period as compared to the control period (Fig. 2).

The acid phosphatase activity was about the same in the two periods (Fig. 2).

The quotient's enzyme activity/DNA and RNA/DNA are shown in Table I. The alkaline phosphatase activity per DNA in the defibrinogenation period is almost twice that of the control period. The quotient RNA/DNA was low in both periods.

Histology. Sponges from the control period showed developing granulation tissue with budding capillaries, fibroblasts and collagen fibrils at the periphery of the sponges and towards the central parts fibrin and inflammatory cells.

The granulation tissue harvested from the defibrinogenation period, showed more inflammatory cells as compared to the control period. The dominating inflammatory cell was the PMN leuco-

cyte. The mononuclear cells were in both periods more sparsely represented. Fibroblasts, collagen fibrils and blood capillaries were only occasionally found, located at periphery of the sponges.

Enzyme histochemistry. The alkaline phosphatase activity was mainly localized in the polymorphonuclear leucocytes (Fig. 3) which were more numerous in the defibrinogenation period. The intensity and localisation of alkaline phosphatase activity in PMN leucocytes per se was about the same in the two periods. Acid phosphatase activity was very weak and no obvious differences could be observed between the two periods.

DISCUSSION

The importance of the initial inflammatory reaction for the subsequent repair process has been known since the studies of Carrel in 1921. The inflammatory period is also sensitive to disturbances, e.g. glucocorticoids and female sex hormones have been shown to delay repair mainly by modifying the inflammatory reaction (see Ahonen, Jiborn & Zederfeldt, 1980). The inflammatory cellular response is

of importance for fibroblast activation (Leibovich & Ross).

Fibrinogen is a major component of the inflammatory exudate (Peacock & van Winkle, 1976). We have recently shown that systemic defibrinogenation interferes with the formation of the initial fibrin framework in a wound (Brändstedt et al.). Delay in development of wound strength and in accumulation of collagen in granulation tissue was also demonstrated (Brändstedt & Olson).

In this study the cell population in early granulation tissue after defibrinogenation was studied.

The inflammatory cells in early granulation tissue contain characteristic and measurable enzymes. Alkaline phosphatase activity has been found suitable to characterize the polymorphonuclear (PMN) leucocytes (Wachstein, 1955; Rank & Skude, unpublished observation). To demonstrate lysosomal enzyme activity mainly contained in macrophages the azo-dye technique for acid phosphatase has been applied (Gedigk & Bontke, 1957). Enzyme histochemistry seems to be of less value to demonstrate fibroblasts in early granulation tissue.

In the histological sections an increased amount of PMN-leucocytes was observed after defibrinogenation. Enzyme histochemistry and biochemistry disclosed increased activity of alkaline phosphatase. Thus the biochemical findings are well correlated to the histological observation. The presence of an increased number of PMN-leucocytes after defibrinogenation in this study on day seven and earlier observations of increased leucocyte accumulation on day four indicates that defibrinogenation increases or prolongs the inflammatory cellular reaction.

Fibrinopeptides are known to generate chemotactic activity of human leucocytes in vitro. However, this has only been demonstrated when using thrombin as the protease. Arvin® induced fibrinopeptides failed to generate any chemotactic activity (Kay, Pepper & Ewart, 1973).

The total number of cells, estimated by measuring the DNA content, is not affected by defibrinogenation. It seems likely that the amount of fibroblasts is reduced in the defibrinogenated period, since the amount of PMN-leucocytes was found increased and no differences in macrophage content could be demonstrated. A reduced amount of active fibroblasts fits into the concept of other findings (Brändstedt, Olson & Ahonen, 1980) where the capacity to synthesize collagen was found de-

creased in granulation tissue after defibrinogenation.

The RNA/DNA quotient has been used as a measure of fibroplasia in wound healing (e.g. Niinikoski, 1969). Fibroblasts contain a high amount of RNA in comparison to the relatively RNA-poor early inflammatory cells and consequently the quotient RNA/DNA rises as the fibroblasts replace the inflammatory cells (Lampiaho & Kulonen, 1967; Ahonen, 1968). We could not in the present study show any difference in this respect between the two periods. The histologically apparent difference is not reflected in the RNA/DNA ratio. However, the quotients are low indicating an immature tissue with a relatively small amount of fibroblasts present during both periods. Any actual differences in number between the fibroblasts present might be disguised by the relatively numerous inflammatory cells. Unpublished data (Brändstedt, 1979) shows that RNA/DNA ratio begins to increase in developing rabbit granulation tissue first after the seventh day.

Measurement of Hb-content in developing granulation tissue has been used as an indicator of the ingrowth of capillaries (Viijanto, 1964; Holmström, Zederfeldt & Ahonen, 1974; Pallin, Ahonen, Rank & Zederfeldt, 1976). It has also been shown that blood supply is necessary for adequate fibroblast proliferation (Silver, 1973). The haemoglobin content is significantly lower after defibrinogenation indicating a delayed capillary ingrowth. This could also be seen in the histological sections.

In conclusion defibrinogenation with Arvin® affects the early inflammatory reaction in granulation tissue formation demonstrated by increased PMN cell infiltration. Concomitantly the development of capillaries and the accumulation of fibroblasts is delayed, which can explain the retardation of healing in Arvin® defibrinogenated animals.

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IV

EFFECT OF DEFIBRINOGENATION ON COLLAGEN SYNTHESIS IN GRANULATION TISSUE

A STUDY IN THE RABBIT

S. BRÄNDSTEDT, P.S. OLSON AND J. AHONEN

ABSTRACT

Collagen synthesis was studied in sponge-induced early granulation tissue from rabbits defibrinogenated by Arvin^R. Tissue slices were incubated *in vitro* with ¹⁴C-proline. The formation of ¹⁴C-hydroxyproline and the incorporation of ¹⁴C-proline into proteins was determined. The incorporation of proline into collagen and proteins was equally reduced after defibrinogenation. A possible explanation for this is a reduced number of active fibroblasts in granulation tissue formed during defibrinogenation.

Defibrinogenation by Arvin^R (a coagulating enzyme from the venom of the Malayan pit viper (*Aghkistrodon rhodostoma*) delays strength development of cutaneous incisions and retards the deposition of collagen in experimental granulation tissue (Brändstedt & Olson, 1980).

The amount of collagen deposited in granulation tissue at a given time is the result of the balance between synthesis and degradation of collagen (Woessner, 1968).

Whether the low amount of collagen observed in early granulation tissue after defibrinogenation (Brändstedt & Olson, 1980) is due to increased lysis or to decreased synthesis has not been decided. The purpose of the present investigation is to study the capacity of granulation tissue formed during defibrinogenation to synthesize collagen and proteins.

MATERIAL

Five rabbits of both sexes, weighing between 2.3 and 4.0 kg, were used. The animals were kept in separate cages and given food pellets (Astra-Ewos, Sweden) and tap water *ad libitum*.

Arvin^R (laboratory grade, batch 6006A7, Twyford Laboratories, London) was used for defibrinogenation.

Viscose cellulose sponge material (Kongfoss Fabrikker, Bygdø, Oslo, Norway) was used to induce granulation tissue formation.

METHODS

Experimental procedure

The rabbits were anesthetized by ether and shaved on one side of the back. Under sterile conditions two skin incisions were made in the shaved area. Through each of the wounds 4 sponges were implanted subcutaneously as described by Viljanto & Kulonen (1962). After 7 days the sponges were removed for analysis under ether anesthesia and the wounds were resutured. The rabbits were allowed to recover for 14 days.

Defibrinogenation was then started with Arvin^R (1 unit/kg body-weight) given twice daily in the marginal ear vein. On the fourth day of defibrinogenation sponges were implanted on the other side of the dorsal midline exactly as was done earlier. The sponges were again removed after 7 days. Defibrinogenation was continued throughout the experimental period.

Determinations of fibrinogen concentration in plasma were made according to Nilsson & Olow (1962) at the time of sponge implantation and removal.

^{14}C -proline incorporation *in vitro*.

The sponges were immediately after removal placed in HEPES buffered Krebs Ringer solution ($+4^{\circ}\text{C}$) (Holmström et al., 1975) and taken to a coldroom where they were freed of remnants of the capsule and cut into 0.5 mm slices with a Stadie Riggs microtome. Tissue slices from two sponges were incubated in 6 ml of the Krebs Ringer solution in a thermostated shaking bath at 37°C for 30 min. 0.74 mBq Proline (10 μl) L- ($^{14}\text{C}(\text{u})$) (New England Nuclear, Boston, Mass.) was added to the solution and incubation was continued for 2½ hours.

The cell activity was terminated by adding 12 ml of 99.5% ethyl alcohol to the incubation mixture. The slices were collected by centrifugation for 30 min. at 4°C and 30 000 g and washed with 70% ethyl alcohol. After drying the sediment to constant weight at room temperature it was homogenized by an Ultra Turrax^R homogenizer in distilled water and dialyzed against running tap water for 48 hours. The dialyzed homogenate was then hydrolyzed in 6 N hydrochloric acid at 130°C for 3 hours. Hydrochloric acid was removed by careful evaporation in a water bath with several washings of distilled water. The residue was dissolved in 15 ml of water. The total radioactivity and the radioactivity of hydroxyproline was determined as described by Juva & Prockop (1966). The recovery of hydroxyproline was 28-48%. Aqueous solutions were counted in a commercial scintillation mixture (Insta-gel^R). Radioactivity was measured in a liquid scintillation counter (LKB Wallac 81 000). At least 1 000 cpm over background were registered. ^{14}C -counting efficiencies were determined by external standardization using a standard quenching diagram prepared from samples obtained from granulation tissue with internal standardization (^{14}C -toluene).

Statistical methods

The significance of the difference between the means was tested with Student t-test for paired observations.

RESULTS

The plasma fibrinogen concentration after defibrinogenation measured at the time of sponge implantation and removal, ranged from immeasurably low to 0.6 g/l.

The results of the incorporation experiment are given in Table I.

The total ^{14}C -proline activity was significantly reduced in the granulation tissue from the defibrinogenation period compared to the activity in granulation tissue from the control period.

TABLE I. Incorporation of ^{14}C -proline into collagen and proteins in 7-day old granuloma slices obtained from Arvin^R-defibrinogenated and control periods from 5 rabbits. Mean $\pm$ SEM.

	^{14}C -Activity in dpm/mg dry weight		ratio ^{14}C -hypro/ ^{14}C -proline
	^{14}C -hypro	^{14}C -proline	
Control	99 $\pm$ 21	1394 $\pm$ 128	0.07
Arvin	40 $\pm$ 14*	667 $\pm$ 165*	0.06

* $P < 0.05$

^{14}C -hydroxyproline activity was significantly reduced in granulation tissue from the defibrinogenation period compared to the activity in granulation tissue from the control period.

The ratio of the ^{14}C -hydroxyproline fraction to the total ^{14}C -proline fraction was the same in granulation tissue from the defibrinogenation period and the control period.

DISCUSSION

Hydroxyproline is a for collagen unique aminoacid, which makes it a useful marker for studies on the metabolism of collagen (Neuman & Logan, 1950). Hydroxyproline cannot be directly incorporated into collagen (Stetten, 1949). Instead, proline is incorporated in the procollagen peptide and is then to a certain percentage hydroxylated to hydroxyproline by a specific enzyme, prolylhydroxylase (Prockop, 1973). This condition makes it possible to study the rate of collagen synthesis by measuring the incorporation of labelled proline into hydroxyproline.

Tissue removed from the organism and placed in suitable buffers will retain metabolic activity for many hours (Holmström et al., 1975; Lampiaho & Kulonen, 1967; Niinikoski, 1969). If radioactively labelled proline is added to such a tissue culture the amount of collagen synthesized during a set period of incubation can be determined by measuring the activity of labelled hydroxyproline. It is assumed that what happens *in vitro* reflects the synthetic capacity of the tissue at the time of harvesting. The *in vitro* method essentially measures the capacity to synthesize collagen under "ideal" conditions, that is the potential capacity of the

enzyme systems, when the supply of oxygen and other substrates is not limiting. Difficulties arising from changed sizes of aminoacid pools are avoided in contrast to *in vivo* incorporation.

The capacity to synthesize collagen was found to be decreased in granulation tissue from the defibrinogenation period as compared to the control period. The delay of collagen accumulation after defibrinogenation observed earlier (Brändstedt & Olson, 1980) can well be explained by a decreased rate of synthesis.

The reduced collagen synthesis can be due either to a direct inhibiting effect on collagen synthesis in the individual fibroblast or to reduced number of collagen producing cells. The present study does not allow definitive conclusions as to the exact mechanism. However, in the defibrinogenation period not only collagen synthesis but also general protein synthesis (measured by incorporation of ^{14}C -proline into all proteins) was reduced. The ratio ^{14}C -hypro/ ^{14}C -proline is not changed by defibrinogenation. Since other cells in early granulation tissue (predominantly leucocytes) synthesize very little protein as compared to fibroblasts, the findings indicate that the reduced collagen synthesis after defibrinogenation is due to a decreased number of active fibroblasts in the granulation tissue. However, this must be evaluated by studies on cell composition of granulation tissue after defibrinogenation.

In conclusion: The reduction of collagen accumulation in granulation tissue that has been observed after systemic defibrinogenation can be explained by decreased collagen synthesis which most likely is due to reduced number of fibroblasts in the granulation tissue.

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V

LACK OF INFLUENCE ON COLLAGEN ACCUMULATION IN GRANULATION
TISSUE WITH "DELAYED" DEFIBRINOGENATION

A STUDY IN THE RABBIT

S. BRÄNDSTEDT AND P.S. OLSON

ABSTRACT

Systemic defibrinogenation using Arvin delays wound strength development and collagen accumulation in sponge induced granulation tissue. Whether this effect is due to interference with the initial fibrin deposition or to any inhibiting action on fibroblast function has not been decided. In the present study the administration of Arvin is started after the initial formation of a fibrin framework. No effect on collagen accumulation in granulation tissue was demonstrated, suggesting that the action of Arvin on wound healing is limited to the first phase of wound healing and that a normal fibrin deposition is necessary for a normal healing process.

The influence of defibrinogenation by Arvin^R, the active coagulant fraction of the venom from the Malayan pit viper (*Agkistrodon rhodostoma*), on wound healing has been investigated in a series of studies in our laboratory. We have found that systemic defibrinogenation delays strength development of cutaneous incisions and retards the deposition of collagen in experimental granulation tissue (Brändstedt & Olson, 1980). Further, a reduced capacity to synthesize collagen and proteins was found in granulation tissue formed during defibrinogenation by Arvin^R (Brändstedt et al., 1980).

The amount of fibroblasts was found to be reduced in granulation tissue after defibrinogenation which offers a likely explanation for the above mentioned findings (Brändstedt et al., 1980). However, a "direct" inhibiting action of Arvin^R on fibroblast function has not been excluded.

The aim of the present study is to find out if Arvin^R, given only during the period of fibroplasia, affects the accumulation of collagen in granulation tissue.

MATERIAL

Eleven rabbits of both sexes, weighing between 2.3 and 4.0 kg were used. The animals were kept in separate cages and fed with food pellets (Astra-Ewos, Sweden) and water *ad libitum*.

Arvin, laboratory grade batch 6006A7 (Twyford Laboratories, London) was used for defibrinogenation.

Viscose cellulose sponge material (Kongfoss Fabrikker, Bygdø, Oslo, Norway) was used to induce granulation tissue formation.

METHODS

Experimental procedure

The rabbits were anesthetized with Veterinary Mebumal (ACO, Sweden) 30 mg/kg body weight and shaved on one side of the back. Under sterile conditions six sponges were implanted subcutaneously through two skin incisions in the shaved area as described by Viljanto & Kulonen (1962). After 7 days the sponges were removed for analysis. The wounds were resutured and the rabbits allowed to recover for 14 days. Sponges were then implanted on the other side of the dorsal midline exactly as was done earlier. Defibrinogenation was started on the second postimplantation day. Arvin^R (1 unit/kg body weight) was given twice daily in the marginal ear vein. Defibrinogenation was continued throughout the experimental period and controlled by determination of the plasma

fibrinogen concentration on the third, fifth and seventh days after implantation. The second set of sponges were removed after 7 days.

Hydroxyproline determination

The carefully decapsulated sponges were homogenized in distilled water with an ultra-Turrax^R homogenizer for 30 seconds. The homogenate was hydrolyzed in 6 N hydrochloric acid for 3 hours at 130° C. The hydrochloric acid was removed by evaporation in a water bath. Hydroxyproline in the hydrolysate was determined as described by Pikkarainen (1968).

Plasma fibrinogen concentration

The plasma fibrinogen concentration was determined according to Nilsson & Olow (1962).

Statistical calculations

Student t-test was used for testing the significance of differences between paired observations.

RESULTS

Plasma fibrinogen concentration was normal at the time of sponge implantation and fell rapidly after the start of defibrinogenation and from the fifth day post implantation it was low (Fig. 1).

Content of hydroxyproline in granulation tissue. No difference in hydroxyproline accumulation was found in sponge induced granulation tissue between the control and the experimental period (Fig. 2).

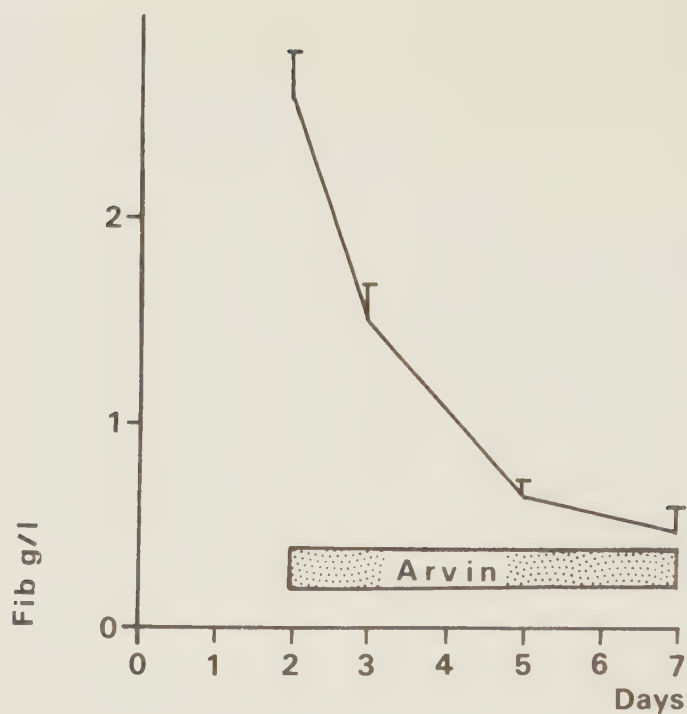


Fig. 1. Fibrinogen concentrations in plasma in rabbits before and after start of defibrinogenation. Mean ($\pm$ SEM) from 11 rabbits.

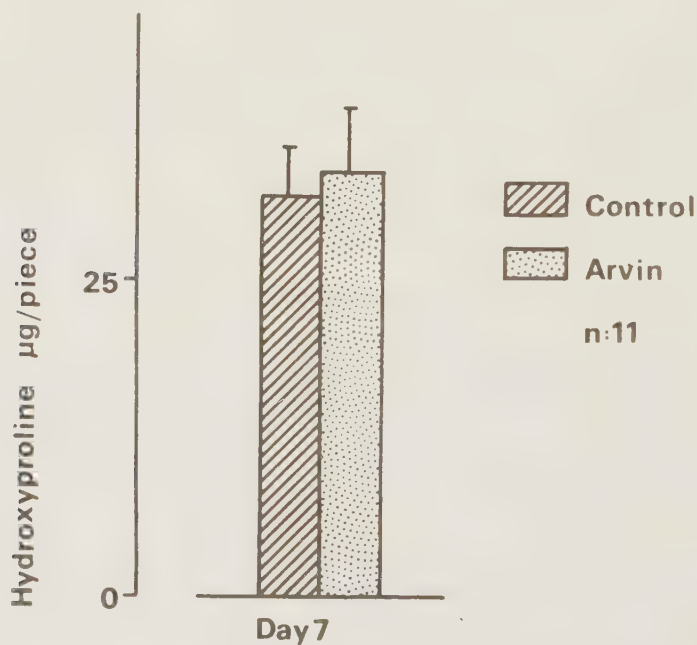


Fig. 2. Hydroxyproline content in sponge induced granulation tissue. Height of columns indicates mean content $\pm$ SEM.

DISCUSSION

The present study was designed to find out whether the impairing effect of Arvin on wound healing is due to an influence on the coagulum or is a direct effect of Arvin on the fibroblast. Viljanto & Kivikoski (1962) have histologically analysed the time sequences of granulation tissue formation in sponge implants. From these studies it is apparent that a dense fibrinous network develops within the pores of the sponges during the first 2 days. In a previous study we have demonstrated that systemic defibrinogenation interferes with the formation of the fibrin framework (Brändstedt et al., 1980). The fibrin deposited in sponges implanted in defibrinogenated animals does not form a dense regular pattern, instead it is sparse and scanty in histological appearance.

Arvin does not possess any fibrinolytic activity and does not activate plasminogen (Straub, 1973; Pizzo et al., 1972). Therefore Arvin should not significantly affect a preformed fibrin clot within the implants in this experimental model.

In the present investigation defibrinogenation was started 2 days after sponge implantation allowing a normal fibrin network to form. Arvin was then given in a dose equivalent to that given in previous studies (Brändstedt & Olson, 1980; Brändstedt et al., 1980) where decreased collagen formation was demonstrated in sponge induced granulation tissue after defibrinogenation.

Cells differentiated into fibroblasts are observed for the first time on the third postimplantation day in sponge induced granulation tissue (Viljanto, 1964). Collagen accumulation can be measured on the fourth day and from the fifth day on there is a sharp increase in collagen production. The collagen accumulation in this study is undisturbed. Therefore the conclusion can be drawn that Arvin does not interfere with the fibroblast function

estimated by the rate of collagen accumulation.

Using the same experimental model we have established that systemic application of Arvin, starting before sponge implantation and given throughout the time of implantation, delays wound healing and reduces the amount of collagen deposited in granulation tissue (Brändstedt & Olson, 1980). We have further found that the collagen synthesizing capacity is reduced after defibrinogenation (Brändstedt et al., 1980).

This investigation indirectly shows that the action of Arvin on wound healing is predominantly limited to the first phase of wound healing. These findings are corroborated by the observations of Daniel et al. (1976). They studied Arvin, used as anticoagulant following endarterectomy in the dog, and observed that animals receiving Arvin only during the first 2 days postoperatively suffered from wound dehiscence in over 50 per cent.

In conclusion, it was found that defibrinogenation, starting 2 days after sponge implantation allowing a normal fibrin deposition to occur, does not affect the early formation of granulation tissue if the fibrin deposition in wound exudate is not disturbed. This means that a normal fibrin framework is necessary for a normal healing process.

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